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Calmodulin kinase IV: expression and function during rat brain development

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Abstract

The expression of calmodulin kinase IV (CaMKIV) can be induced by the thyroid hormone T_3 in a time- and concentration-dependent manner at a very early stage of brain differentiation using a fetal rat telencephalon primary cell culture system which can grow and differentiate under chemically defined conditions (Krebs et al. (1996) J. Biol. Chem. 271, 11055–11058). After the induction of CaMKIV by T_3 we examined the influence of prolonged absence of T_3 from the culture medium on the expression of CaMKIV. We could demonstrate that after the T_3-dependent induction of CaMKIV, omission of the hormone, even for 8 days, from the medium did not downregulate the expression of CaMKIV indicating that different regulatory mechanisms became important for the expression of the enzyme. We further showed that CaMKIV could be involved in the Ca^{2+}-dependent expression of the immediate early gene c-*fos*, probably via phosphorylation of the transcription factor CREB. Convergence of signal transduction pathways on this transcription factor by using different protein kinases may explain the importance of CREB for the regulation of different cellular processes.

Keywords: Calmodulin kinase; Calmodulin; Thyroid hormone T_3; Immediate early gene; c-*fos*; Development; (Rat brain)

1. Introduction

Calcium ion is one of the most versatile second messengers involved in cell growth and differentiation [1]. It plays its pivotal role through a specific class of proteins, the so-called EF-hand type Ca^{2+}-binding proteins [2], the most prominent representative of which is calmodulin (CaM) [3]. As an intracellular calcium-sensor calmodulin binds to and activates a great variety of enzymes, most notably a class of different protein kinases and phosphatase(s) – i.e., CaMkinases and calcineurin. Since protein kinases and phosphatases are key modulators of cellular regulatory processes, it is not surprising that CaMkinases are involved in the regulation of such central processes as neurotransmitter release, muscle contraction, cell proliferation and gene expression (for reviews see Refs. [4] and [5]). Six different CaM-dependent kinases are known to date, all belonging to the class of enzymes phosphorylating serine-threonine residues. The targets can be either of multiple sources (e.g., CaMkinase I, II and IV) or dedicated to single substrates (e.g., myosin light chain kinase, phosphorylase kinase or CaMkinase III).

CaMkinase IV (CaMKIV) was first described as being particularly abundant in the granular cells of the cerebellum [6]. Therefore, the enzyme was called CaMkinase 'Gr' [6], but later the protein was renamed CaMkinase IV (CaMKIV) [7,8]. Next to nervous tissues CaMKIV is strongly expressed in the thymus, particularly in T-lymphocytes [9] and, to a somewhat lower extent, in the spleen and in testis, but is undectable in all other tissues examined [6,7,10,11].

The gene structure of CaMKIV has been reported recently [12]. The enzyme is expressed in two spliced isoforms of M_r 65 000 (α) and of M_r 67 000 (β), the latter being found mainly in the cerebellum [13]. The primary structure has been obtained by cDNA cloning from rat, mouse and human brain libraries [7,8,14–16]. The regula-

Abbreviations: CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinaseII; CaMKIV, Ca^{2+}/CaM-dependent protein kinase IV or Gr; CREB, cAMP response element binding protein; IEG, immediate early gene; PBS, phosphate-buffered saline; SRF, serum response factor; T_3, 3,3',5-triiodo-L-thyronine.

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tory and catalytic domains of CaMKIV are highly homologous to CaMKII, but the overall homology between the two enzymes is less than 50%.

The substrate specificity of CaMKIV seems to be somewhat restricted. Next to synapsin I [6] and the Ras related GTP-binding protein Rap1b [17] mainly transcription factors such as the cAMP regulatory element binding protein CREB [18,19], the serum response factor SRF [20,21] or members of the Ets family of transcription factors [22,23] could be phosphorylated by CaMKIV. Since CaMKIV has recently been reported to be located also in the nucleus [18,24], the enzyme could have direct access to transcription factors to regulate their function in a Ca^{2+}-dependent manner. Thus it has been shown in different cell lines that CaMKIV is involved in the regulation of expression of IEGs either through CREB [18,19,25,26] or through SRF [21].

Since it was originally assumed that CaMKIV like CaMKII is activated by an autophosphorylating mechanism, the recent reports that autophosphorylation of CaMKIV was a slow and inefficient process [19,27], was a puzzling observation. Recombinant CaMKIV expressed either in *Escherichia coli* [28,29] or in baculovirus/Sf9 cells [27,30] was activated by autophosphorylation only 2-fold, even after incubation for 1–2 hours. This activation could be significantly improved by preincubation with brain extracts [28–30] out of which subsequently a CaMkinase kinase could be purified and characterized [30–32]. This Ca^{2+}/CaM-dependent enzyme of 66–68 kDa [30] could activate CaMKIV upto 30-fold within less than 1 min [32,33]. Thus, CaMKIV involved in the regulation of gene transcription could be activated through a CaMkinase cascade, reminiscent of the regulation of mitogen activated protein (MAP)-kinase activity.

Growth and differentiation of the mammalian brain has been shown to be controlled by different growth factors and hormones, among them the thyroid hormone 3,3',5-triiodo-L-thyronine T_3 [34,35]. T_3 acts through a specific receptor recognizing regulatory elements of the expressed gene like it has been described for other members of the superfamily of steroid/thyroid hormone receptors [36]. Recently, we reported the specific induction of CaMKIV by T_3 in a time- and concentration dependent manner [37]. This induction occurred at a very early stage of brain differentiation using a fetal rat telencephalon primary cell culture system which can grow and differentiate under chemically defined conditions [38]. It could be shown that the induction was T_3-specific – i.e., the expression of CaMKIV could not be induced by either reverse T_3 or retinoic acid [37]. The expression of CaMKIV was regulated both on the transcriptional level and on the translational level; e.g., the addition of actinomycinD as well as cycloheximide to the culture medium could prevent the T_3-dependent induction of the enzyme. In addition, the T_3-specific expression could be observed both on the mRNA and on the protein level [37]. Here we report that CaMKIV could be involved in the regulation of expression of IEGs as it has been proposed by different Laboratories [18,25,39]. We could show that the expression of c-*fos* was significantly reduced provided T_3 (i.e., CaMKIV) was absent from those cultures. This reduction in the expression of c-*fos* occurred only if the cells were depolarized by adding KCl to the medium to promote Ca^{2+}-influx. On the other hand, activation of the cells through other pathways – e.g., through protein kinase C – provided no difference in c-*fos* expression by comparing cultures grown in the presence or absence of T_3. Furthermore, we could demonstrate that the expression of CaMKIV, once started by adding T_3, could not be downregulated by removing T_3 from the medium as, by contrast, has been demonstrated, e.g., for myelin basic protein [40]. These findings suggest that the induction of CaMKIV by T_3 during rat brain development may be a terminal switch (i.e., CaMKIV could be instrumental in brain differentiation) possibly through the Ca^{2+}-dependent regulation of expression of IEGs.

2. Materials and methods

2.1. Cell culture

Serum-free, rotation-mediated aggregating cell cultures were prepared from fetal (15 days of gestation) rat telencephalon, as described in detail previously [37,38]. The culture medium used was Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Gaithersburg, MD, USA), supplemented with nutritional factors, vitamins, trace elements, transferrin (1 mg/l), insulin (800 nM), and hydrocortisone-21 phosphate (20 nM). Gentamicin sulfate (25 mg/l) was used as an antibiotic. For analyses, the aggregates of each flask were washed twice with 5 ml of phosphate-buffered saline (PBS) and proteins were extracted as described in [37].

2.2. Extraction of total cellular RNA and Northern blot analysis

Total RNA was extracted according to the procedure of Gerstenfeld et al. [41] as described in detail elsewhere [42]. Briefly, after washing cell culture aggregates (grown for 5 days either in the presence or absence of 3×10^{-8} M T_3) with ice-cold phosphate-buffered saline the cells were lysed in a buffer containing 4 M guanidinium isothiocyanate, 25 mM sodium acetate and 120 mM β-mercaptoethanol and sheared by 5 passages through a 21 gauge needle. Total RNA was purified by ultracentrifugation through cesium chloride as described by Chirgwin et al. [43]. RNA samples (10 μg aliquots) were denatured by heating for 10 min at 55°C, electrophoretically separated on a 1.4% agarose/formaldehyde gel, transferred to Gene Screen filters by capillary blotting, and cross-linked to the

filter by exposure to UV light for two minutes. After pre-hybridization for 6 hours at 65°C [42] the filters were hybridized with ^{32}P-labeled probes (10^6cpm/ml) by incubation overnight at 65°C. SP6 recombinant transcripts of v-*fos* (685 bp *Sal*I to *Pst*I; [44]) and glyceraldehyde-phosphate dehydrogenase (GAPDH; 1400 bp *Pst*I; [45]) were used as riboprobes for the hybridization. After several washings [42] the filters were exposed to Amersham Hyperfilm for 30 h at −80°C using an intensifying screen. The levels of c-*fos* mRNA were determined by scanning densitometry, and normalized for differences in gel loading relative to the levels of GAPDH mRNA determined by rehybridization after stripping of the filters as described before [42].

2.3. Identification of CaMKIV

In order to identify and characterize CaMKIV, aggregates of a total of 20 culture flasks (ca. 10^9 cells), grown for 5 days in the presence of T_3 (3×10^{-8}M) have been collected, washed twice with ice-cold PBS and homogenized at 4°C in 3 vols. of ice-cold buffer containing 25 mM Hepes (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM PMSF, 1 mM leupeptine, 1 mM TPCK, 1 mM TLCK, 1,10-Phenantroline, 1 mM *p*-aminobenzamidine. CaMKIV was isolated and identified as described in detail in Ref. [37]. Calmodulin-dependent kinase activity was characterized by autophosphorylation using the assay as described by Cruzalegui and Means [27]. Identification of CaMKIV by specific antibodies kindly provided by Dr. A.R. Means, Durham, USA, was carried out by Western blotting using 2% milk powder/PBS as blocking buffer.

2.4. SDS-PAGE and electrophoretic blotting

After extraction of the proteins from the pellet of whole aggregates ($4–5 \times 10^7$ cells) as described in detail in [37], proteins were separated by SDS-PAGE [46] and electrophoretically blotted onto nitrocellulose sheets. CaM-binding proteins were identified on the blots by incubation with ^{125}I-labeled CaM [47] and exposed to PhosphoImager screens as described before [37]. Quantitation was obtained using the PhosphoImager software as recommended by the manufacturers.

3. Results

As described in detail in [37] the expression of CaMKIV in fetal rat brain primary tissue cultures is induced by the thyroid hormone 3,3',5-triiodo-L-thyronine (T_3) in a dose and time dependent manner. Therefore, aggregating cell cultures from 15-day fetal rat telencephalon [37] were incubated for 5 days with increasing amounts of T_3. After collection of the cell pellets the proteins were extracted and separated by SDS-polyacrylamide gel electrophoresis,

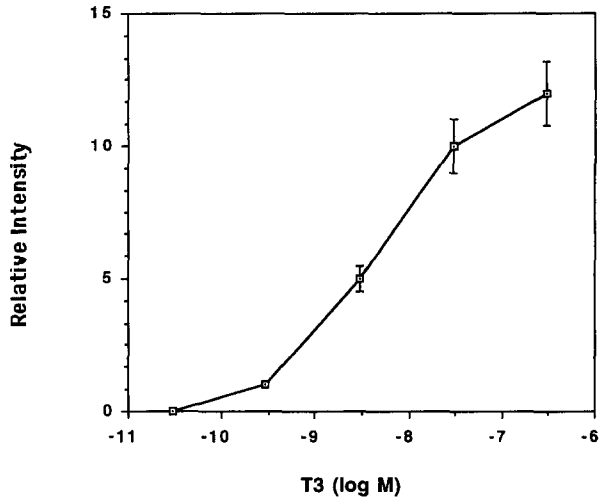

Fig. 1. Quantification of the dose-dependent expression of CaMKIV in response to different T_3 concentrations as described in Section 2; see also Ref. [37]. The values are given as relative intensities, setting the value for 3×10^{-10} M T_3 to 1.

electrophoretically transferred onto nitrocellulose and CaM-binding proteins were identified by incubation with ^{125}I- labeled CaM [37]. CaMKIV was detectable already at very low concentrations of T_3 (3×10^{-10} M; Ref. [37]) and increased in intensity with increasing concentrations of T_3 in the culture medium, indicating that the induction of CaMKIV by T_3 was dose-dependent. As demonstrated in Fig. 1, quantitation of those results indicated a more than 10 fold increase of CaMKIV expression by comparing the amount of protein induced by different T_3 concentrations. The induction of CaMKIV protein synthesis by T_3 was paralleled on the transcriptional level – i.e., CaMKIV mRNA was detectable only in cell cultures incubated with T_3 as shown in [37].

If the mRNA and protein synthesis of CaMKIV were totally dependent on the presence of T_3, then removal of T_3 from the culture medium should downregulate the synthesis of CaMKIV. To test this hypothesis, cultures

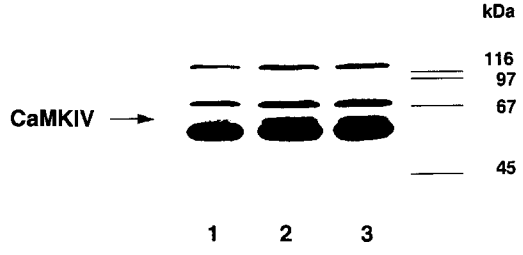

Fig. 2. Prolonged expression of CaMKIV after removal of T_3 from the culture medium. ^{125}I-CaM overlay of extracts of fetal rat telencephalon cultures grown for 13 days in the absence (lane 1) or in the presence (lane 2) of 3×10^{-8} M T_3. Overlays of lane 3 have been made with extracts of cultures grown first for 5 days in the presence of 3×10^{-8} M T_3 and afterwards for 8 days in the absence of the hormone. Culture media were exchanged every other day. CaMKIV was identified as described in detail in Ref. [37]. The unresolved bands immediately below CaMKIV have been identified as Calcineurin A and CaMKIIβ, respectively.

grown for 5 days in the presence of 3×10^{-8} M T_3 were exchanged with media not containing T_3 and cells were harvested at different time intervals. As shown in Fig. 2 CaMKIV could be detected, even if the cultures have been incubated up to 8 days with media lacking T_3 indicating that CaMKIV synthesis becomes independent of T_3, once it had been induced by the hormone. Similar results could be obtained if the cultures had been analyzed already after 3 or 5 days (data not shown). This is in contrast to findings obtained with the myelin basic protein [40] which is downregulated after removal of T_3 from the culture medium for 24 h. These rather surprising results suggest that after the induction of CaMKIV by T_3 which is essential for the initiation process as documented earlier [37], a different regulatory mechanism is dominating, indicating that probably a different responsive element becomes accessible after the induction by T_3. This could be under the control of a kinase, since preliminary experiments with the kinase inhibitor staurosporine seem to indicate a downregulation of CaMKIV in the presence of this inhibitor.

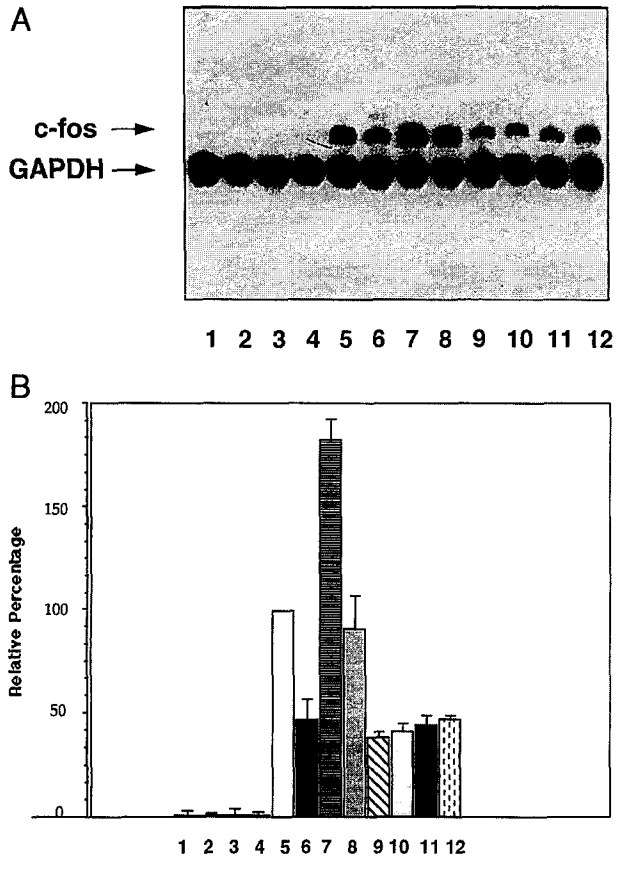

Fig. 3. Influence of CaMKIV on the expression of c-*fos*. (A) Autoradiograms of gene screen filters after electroblotting, hybridization with probes for c-*fos* and GAPDH and exposure to film as described in detail in the Section 2. To each lane 10 μg aliquots of RNA has been applied. The following conditions have been used for the different experiments: Cultures have been grown for 5 days either in the absence (lanes 2, 4, 6, 8, 10, 12) or in the presence (lanes 1, 3, 5, 7, 9, 11) of 3×10^{-8} M T_3. Lanes 1–4, controls, no stimulation; lanes 5–12, stimulation by incubation of the cultures for 1 hour with either 30 mM KCl (5–8) or with 100 nM mezerein (9–12). To cultures of lanes 7, 8, 11, 12 1-β-D-arabino-furanosylcytosine (Ara-C) has been added to prevent proliferation of glia cells and to enrich the cultures with neuronal tissue. The shown autoradiogram is a representative of five different experiments. (B) Densitometric quantification of data as shown in (A). The data were normalized relative to the data obtained for GAPDH and the value obtained with 30 mM KCl in the presence of T_3 (lane 5) was set to 100%. The data given in the figure are an average of five different experiments, the error deviation is indicated. The numbers of the different lanes correspond to those of (A).

As outlined before, CaMKIV may be involved in the transcriptional regulation of IEGs through the transcription factor CREB, originally identified as the cAMP regulatory element binding protein. If this were the case, then cell cultures grown in the presence or absence of T_3 should demonstrate a significant difference in the level of c-*fos* expression, which has been shown to be dependent on CREB mediating Ca^{2+} responses [5]. As shown in Fig. 3 the level of c-*fos* mRNA differed significantly depending on the process of stimulation. If the cells have been incubated with 100 nM mezerein for 1 hour to stimulate a protein kinaseC pathway, the level of c-*fos* was practically identical comparing cultures grown in the presence or absence of T_3. However, by incubating the cells with 30 mM KCl to achieve membrane depolarization and subsequent Ca^{2+}-influx into the cells a significant decrease of c-*fos* induction could be noticed in the absence of T_3 (i.e., in the absence of CaMKIV), suggesting that CaMKIV may be involved in the Ca^{2+}-dependent stimulation of c-*fos* expression. This difference in c-*fos* expression became even more visible if the cultures had been pretreated with 1-β-D-arabino-furanosylcytosine (Ara-C) to prevent proliferation of glia cells and to enrich the cultures with neuronal tissues, since CaMKIV is highly expressed in neurons as described before [37]. As shown in Fig. 3B, by normalizing the expression of c-*fos* to the level of GAPDH expression, less than 50% of c-*fos* were expressed in the absence of CaMKIV if the cells were stimulated by membrane depolarization, whereas no difference could be noticed if the cells had been stimulated in a Ca^{2+}-independent manner.

4. Discussion

In this and in an earlier contribution [37] we have provided compelling evidence that CaMKIV is induced by the thyroid hormone T_3 during the early stages of embryonal brain development. The expression of the enzyme is dose- and time-dependent, is controlled on the transcriptional level and is T_3 specific – i.e., neither reverse T_3 nor retinoic acid nor other growth factors can stimulate CaMKIV expression. The kinase may be specifically expressed in neurons, since the enzyme was found at high levels in neuron-enriched aggregate cultures in which highly proliferating glial cells have been suppressed by the addition of 1-β-D-arabino-furanosylcytosine (Ara-C) [48].

It was interesting to note that the expression of CaMKIV could be induced by the addition of T_3 to the culture medium, but that removal of T_3 did not turn off the synthesis of the enzyme. This is typical for a terminal switch during cell differentiation, similar to observations during muscle differentiation [49]. Recent experiments seem to indicate that differentiation processes of neurons in these cell aggregates were significantly reduced if CaMKIV was either inhibited or not expressed (Krebs and Honegger, unpublished observations). This was especially noticeable for cholinergic neurons in contrast to GABAergic neurons or to glia cells.

In the developing brain, synaptic connectivity of neurons may be critically dependent on rapid genomic responses to neuronal stimulation. A group of genes that is rapidly and transiently activated by growth factors and other stimulatory mechanisms are referred to as 'immediate early genes' (IEGs), a member of which is c-*fos*. A number of IEGs code for transcription factors such as members of the *fos* and *jun* families, which dimerize to form the transcription regulatory complex AP-1. Neuronal stimulation, both in vitro and in vivo, can activate c-*fos*, as well as other IEGs encoding transcription factors controlling the expression of late response genes important for neuronal plasticity and excitability [50]. There is ample evidence that several signal transduction pathways can enhance the expression of c-*fos*, the best characterized system modulating the expression through the transcription factor CREB [51]. As indicated before, CREB binds to the cAMP responsive element (CRE) of several genes, and initiation of gene transcription requires phosphorylation of Ser 133 [52]. CREB can be phosphorylated not only by cAMP dependent protein kinase [52], but also by CaMkinases [18,19,39] thereby suggesting a mechanism to explain the Ca^{2+}-dependent regulation of expression of CRE-containing genes such as c-*fos* [39]. In the present paper we provided evidence that CaMKIV could be involved in the regulation of c-*fos* expression in a primary brain tissue culture system, possibly through the phosphorylation of CREB, since Ca^{2+}-dependent stimulation of c-*fos* expression was downregulated in the absence of CaMKIV (Fig. 3). Of course, the given evidence is only indirect and other explanations could be possible, but the conclusions drawn upon the results described before are the most straightforward. Similar observations have been made by Enslén and Soderling [25] reporting that in PC12 cells Ca^{2+}-dependent expression of c-*fos* and other IEGs were modulated by a member of the CaMkinase family since calcium-dependent transcription could be inhibited by the CaMkinase-specific inhibitor KN-62. In this context it is of interest that Matthews et al. [18] by using transfected JEG-3 cells provided evidence that only CaMKIV, but not CaMKII, was able to transactivate a CRE-containing reporter gene in transient expression assays through phosphorylation of CREB. The authors provided evidence that the difference in activation may depend on the ability

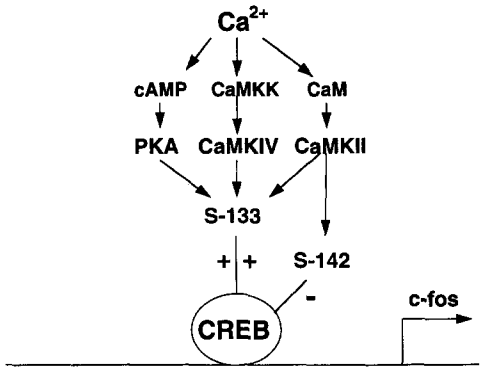

Fig. 4. Schematic view of different signal transduction pathways converging on CREB. The scheme summarizes the influence of different kinases on CREB phosphorylation involved in different Ca^{2+}-dependent pathways as discussed in the text, and is based also on observations described in Refs. [18,39,52]. The following abbreviations have been used: CaMKII,IV = calmodulin-dependent kinase II,IV; CaMKK = calmodulin-dependent kinase kinase; CREB = cAMP-dependent regulatory element binding protein; PKA = cAMP-dependent protein kinase. The signs (+) indicate upregulation and (−) downregulation of the c-*fos* expression.

that only CaMKIV could translocate to the nucleus [18]. In addition, Sun et al. [26] reported recently that CaMKII could phosphorylate CREB not only on residue Ser-133, but also on Ser-142, the latter having an inhibitory influence on CREB activity. This may explain the recent finding of Nghiem et al. [53] who reported that in Jurkat T-cells transfected with a constitutively active mutant of CaMKII, expression of interleukin-2 (Il-2) was downregulated by more than 90%. Since Il-2 expression is mainly controled via the transcription factors NFAT and AP-1, the latter being a heterodimer of c-*Fos* and c-*Jun*, it could be suggested that downregulation of Il-2 could occur through inhibition of CREB controlling c-*Fos* expression. It is interesting that Nghiem et al. [53] indeed found that CaMKII significantly decreased stimulated transcription from AP-1. In Fig. 4 we provide a schematic view of the different signal transduction pathways which may converge at the phosphorylation and subsequent activation or inhibition of CREB.

In conclusion, our results indicate that during rat brain development the expression of CaMKIV, not detectable at the early stages of ontogenesis (i.e., at E15), is induced by the thyroid hormone in a time- and concentration-dependent manner, but once the gene has been turned on, the expression becomes T_3-independent suggesting that an additional regulatory mechanism is involved in CaMKIV expression. Whether this T_3-dependent regulation is due to a direct interaction of the T_3-receptor with a responsive element of the CaMKIV gene [36] or whether the effect is indirect remains to be determined. Furthermore, our results indicated that the Ca^{2+}-dependent expression of the IEG c-*fos* could, at least partly, be regulated by CaMKIV, since the expression of c-*fos* was more than 50% reduced in the absence of CaMKIV. Since CaMKIV seems to play an important role in rat brain development, as our results

indicate, it will be now of interest to know, whether in the immune system CaMKIV is under the control of the thyroid hormone, too, or whether different signals regulate the expression. Preliminary results indicate that the synthesis of CaMKIV in the rat embryonal thymus is induced following the same time-course as in the embryonal brain.

Acknowledgements

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